Attorney's Docket No. 035052/204373 (5052-53)

PATENT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

In re:	Walsh *et al.*	Confirmation No.:	7095
Application No.:	09/689,430	Group Art Unit	1632
Filed:	October 12, 2000	Examiner	Q. Li

For: ADENO-ASSOCIATED VIRUS VECTORS ENCODING FACTOR VIII AND METHODS OF USING THE SAME

Commissioner for Patents
Washington DC 20231

DECLARATION UNDER 37 CFR 1.131

I, Hengjun Chao, do hereby declare as follows:

1. I am an inventor of the patent application identified above and the subject matter described and claimed therein.

2. Prior to October 20, 1998, having earlier conceived of expressing FVIII using AAV vectors, we reduced to practice the subject matter claimed in the present application. Evidence of this work is set forth in Exhibits A and B. These exhibits are pages from my laboratory notebook, containing data from experiments conducted prior to October 20, 1998.

The group of experiments described in Exhibit A show the construction of pDLZ2, an AAV vector containing a 3' UTR truncated B-domain deleted (BDD) human factor VIII (hFVIII), and the HSV thymidine kinase (TK) promoter and poly A sequence from pMC1neoPolyA (Stratagene® Inc., La Jolla, CA) inserted within the 2 ITR's of AAV from pTR-UF5 (description available at hwww.gtc.ufl.edu/ptr-uf5.htm). The

strategy involved using the polymerase chain reaction (PCR) to amplify the BDD hFVIII sequence. The resulting PCR product was inserted into the cassette in a position upstream of the HSV polyadenylation sequence and flanked by the AAV ITRs.

The pDLZ2 vector was subsequently used to produce rAAV virion particles by a three plasmid transfection. The vector containing the BDD hFVIII insert and AAV helper plasmids pXX2 and pXX6 (described in Xiao *et al.* (1998) *J. Virology* 72: 2224-2232) were transfected into 293 cells using calcium phosphate precipitation, and the virus particles were subsequently harvested, purified, and concentrated by cesium density gradient centrifugation.

The pDLZ2 virus particles were used to transduce 293 cells, and FVIII levels were detected by Enzyme-Linked Immunosorbent Assay (ELISA). For the ELISA assay, 96-well plates were coated with monoclonal sheep anti-hFVIII. The hFVIII protein present in the media was detected by a secondary peroxidase-conjugated sheep anti-hFVIII antibody. FVIII levels were calculated according to a standard curve produced using pooled normal human plasma. Function of the rAAV-originated BDD hFVIII was tested by the activated partial thromboplastin time (APTT) assay and the Coatest (Chromgenix AB, Sweden) assay. The APTT assay is a quantitative assay for monitoring procoagulation activity. Briefly, FVIII-deficient plasma, silicate particles, and the serum or cell medium was mixed and incubated at 37°C for exactly 3 min. Upon the addition of $CaCl_2$, the clot forming time was recorded by a fibrometer. Normal human plasma was used to generate the standard curve. The Coatest assay is designed to determine the amount of heparin and FVIII in plasma by adding antithrombin to the sample, which forms a complex with the heparin. Factor IXa (FIXa) is then added to the sample, is

activated by FVIII, and is subsequently neutralized in proportion to the amount of heparin and FVIII present in the sample. The remaining (unneutralized) FIXa then hydrolyzes a chromogenic substrate, allowing determination of the amount of heparin by photometric detection and comparison to a standard curve. Low levels of BDD hFVIII were found to be released from the pDLZ2-transduced cells.

In order to increase levels of BDD hFVIII expressed, an enhancer element from hepatitis B virus (HBV) was cloned into the expression cassette, creating pDLZ6, which lacks the HSV TK promoter. Because the pDLZ6 vector is derived from pDLZ2, the BDD hFVIII coding sequence for these vectors is identical. As a negative control, the BDD huFVIII cDNA in pDLZ6 was replaced with EGFP cDNA from pTR-EGFP to construct pDLZ8 (which also lacks the TK promoter). Virus particles were produced and HepG2 and rat hepatocytes were transduced with these virus particles. APTT and Coatest assays were performed as described above and these assays confirmed the expression of functional BDD hFVIII. The construction of the pDLZ6 and pDLZ8 vectors and the expression of functional BDD hFVIII in cells transduced with pDLZ6 are shown on pages 93-152 of Exhibit A and pages 1-6 of Exhibit B.

3. All of the work described in Exhibit A took place in the United States.

4. Each of the entry dates deleted from experiments in Exhibit A is prior to October 20, 1998.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Hengjun Chao

6/12/2002
Date